Molecular Pathways: The Role of NR4A Orphan Nuclear Receptors in Cancer

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Running Title: NR4A Receptors in Cancer

Abbreviations:

ACTH=Adrenocorticotrophic hormone
MINOR= mitogen inducible orphan nuclear receptor
NGF1-B= nerve growth factor 1 beta
NOR-1= neuron derived orphan receptor 1
NR4A= Nuclear Receptor 4 A
TR3= Testicular Receptor 3
NURR-1= Nur related factor 1
PARP-1= Poly [ADP-ribose] polymerase
PGC1α= peroxisome proliferator-activated receptor gamma co-activator 1 alpha
PPAR-γ= peroxisome proliferator-activated receptor gamma
RAR= Retinoic Acid Receptor
ABSTRACT

Nuclear receptors are of integral importance in carcinogenesis. Manipulation of classic ligand activated nuclear receptors, such as oestrogen receptor blockade in breast cancer, is an important established cancer therapy. Orphan nuclear receptors, such as Nuclear Family 4 Subgroup A (NR4A) receptors, have no known natural ligand(s). These elusive receptors are increasingly recognised as molecular switches in cell survival, and a molecular link between inflammation and cancer. NR4A receptors act as transcription factors, altering expression of down-stream genes in apoptosis (Fas-Ligand, TRAIL), proliferation, DNA repair, metabolism, cell migration, inflammation (II-8) and angiogenesis (VEGF). NR4A receptors are modulated by multiple cell-signalling pathways including Protein Kinase A/CREB, NFkB, PI3K/AKT, JNK, Wnt and MAP kinase pathways. NR4A receptor effects are context and tissue specific, influenced by their levels of expression, posttranslational modification and interaction with other transcription factors (RXR, PPAR-). Subcellular location of NR4A “nuclear receptors” is also important functionally; novel roles have been described in the cytoplasm where NR4A proteins act both indirectly and directly on the mitochondria to promote apoptosis via Bcl-2. NR4A receptors are implicated in a wide variety of malignancies including breast, lung, colon, bladder and prostate cancer, glioblastoma multiforme, sarcoma and acute/chronic myeloid leukaemia. NR4A receptors modulate response to conventional chemotherapy and represent an exciting frontier for chemotherapeutic intervention as novel agents targeting NR4A receptors have now been developed. This review provides a concise clinical overview of current knowledge of NR4A signalling in cancer, and the potential for therapeutic manipulation.
BACKGROUND

Targeting ligand activated steroid nuclear receptors is an important established cancer therapy. Orphan nuclear receptors are similar to steroid nuclear receptors as they act as transcription factors to modulate down-stream gene expression. However, orphan nuclear receptors have no known natural ligand(s). These intriguing receptors comprise over half the total number of nuclear receptors. NR4A orphan nuclear receptors NR4A1 (Nur77; TR3; NGFB-1), NR4A2 (NURR1) and NR4A3 (NOR-1; MINOR) are thought to be incapable of classic ligand binding due to bulky ligand binding domains, unlike “adopted” receptors, e.g. PPAR-γ (NR1C3), where putative ligands have since been discovered. Diverse and paradoxical transcriptional and direct roles have been described for these receptors; physiological functions of NR4A receptors are context and tissue specific. NR4A receptors have emerged as important molecular switches in processes associated with carcinogenesis, including apoptosis, DNA repair, proliferation, migration, inflammation, metabolism and angiogenesis. (Figure) Unlike classic steroid hormone receptors which need to be activated by a ligand, these proteins are constitutively active. (1) Emerging techniques using cell-specific knockdown of NR4A receptors in vivo has recently accelerated discovery of NR4A receptor functions. (2) The actions of NR4A receptors depend on NR4A receptor subcellular localisation, levels of NR4A expression, transcriptional modulation by co-activators/co-repressors, post-translational modification and interaction with other nuclear receptors. (3, 4)

NR4A receptors and Cancer

Apoptosis

Inhibition of Apoptosis

NR4A receptors can promote cell growth and survival, activating transcription of downstream anti-apoptotic and pro-proliferative genes. Physiologically, NR4A2 is essential for dopaminergic neuronal survival in the central nervous system, and reduced NR4A2 is implicated in
Parkinson’s disease. (5) In breast cancer in vitro, NR4A2 inhibits p53-mediated induction of downstream pro-apoptotic genes like BAX, a pro-apoptotic member of the Bcl-2 family. (6) NR4A2 also inhibits apoptosis via convergence with Wnt and MAP kinase pathways. (7, 8) Kitagawa et al have reported that β-catenin binds NR4A2, releasing NR4A2 from the co-repressor protein, Lef-1, in 293F cells, allowing transcription of downstream Wnt and NR4A2 targets. Physiologically, these interactions are required for normal neuronal development and the survival of dopaminergic neurons. (7) NR4A receptors and β-catenin modulate each other’s transcriptional activity in a cell-specific manner. (7, 9) In colon cancer cell lines, a bile acid carcinogen (deoxycholic acid) has been shown to stimulate β-catenin dependent increased expression of NR4A1. (10) Conversely, NR4A1 has been shown to promote degradation of cytoplasmic β-catenin in a transcription-independent mechanism, while in murine models NR4A1 has been shown to reduce tumour cell proliferation by transcriptional inhibition of Wnt signalling. (11, 12)

Proapoptotic Roles for the NR4A receptors

Proapoptotic roles have also been described for the NR4A receptors. Nuclear export of NR4A1 is important functionally as cytosolic NR4A1 has a non-genomic proapoptotic role in cancer cell lines in vitro. NR4A1 induces apoptosis by direct interaction with Bcl-2 in the mitochondria, exposing proapoptotic BH3, or indirectly by stimulating other cytosolic proapoptotic proteins, such as BAX binding to the mitochondria to initiate the apoptotic cascade. (13, 14) (15, 16) (Figure 1 A) Bcl-2 modulation by NR4A1 is also important physiologically in the negative selection of T cells. (13) Nuclear NR4A receptors can also have pro-apoptotic effects by inducing proapoptotic and anti-proliferative genes (1, 17)

DNA repair

A novel function for NR4A receptors in DNA double stranded break repair has recently been identified. (18) NR4A receptors translocate to sites of double stranded DNA damage in a mechanism dependent on PARP-1, and are phosphorylated by DNA protein kinases. Interestingly,
the DNA repair action of NR4A receptors is not dependent on their transcriptional activity, but rather due to a direct interaction at the DNA repair site, the precise mechanisms of which remain incompletely understood.(18) In melanoma, repression of NR4A receptors impairs UV-induced DNA damage repair via the melanocortin-1 receptor. (19) These studies suggest a pro-cell survival role for NR4As by regulating DNA repair. Conversely, a role for NR4A1 in inhibition of DNA repair has been described in hepatocellular carcinoma cell lines. (20)

**Inflammation**

NR4A receptors are an important molecular link between inflammation and cancer. Pro-inflammatory effects of NR4A receptors are seen in the tumour-like growth of pannus in rheumatoid arthritis, where inflammatory synovial hyperplasia becomes an invasive front of destructive tissue causing cartilage damage. (3) Conversely, anti-inflammatory effects have been demonstrated in transformed normal vascular macrophages, endothelial cells in atherosclerosis and in the central nervous system.(21, 22) Pro-inflammatory prostaglandins are strongly implicated in cancer; PGE$_2$ induces NR4A2 in colon cancer, which leads to a myriad of downstream pro-cancer effects. Meanwhile, COX-2 inhibitors repress NR4A2 expression and NR4A regulated genes including osteopontin. (23) This may represent a mechanism for the reduced colon cancer risk observed in population studies of non-steroidal anti-inflammatory drugs (NSAIDS). NR4A receptors are downstream targets of the cyclic AMP response element binding protein (CREB); binding sites for CREB have been identified in the promoter region of all 3 NR4A receptors. Prostaglandin E2 induction of NR4A receptors involves CREB and NFkB signalling, as PGE2 induces phosphorylation of CREB; this phosphorylated CREB can then bind to NR4A promoters and enhance gene transcription.(23)

**Metabolism and Angiogenesis**

NR4A receptors are involved in fatty acid oxidation and hepatic glucose metabolism.(24) In normal skeletal muscle physiology, NR4A3 has been shown to promote fatty acid oxidative pathways, and is induced by β-adrenergic signalling via Protein Kinase A, MAPKinase and CREB dependent pathways. (25, 26) In colon cancer, NR4A2 with PGC1α induces expression of fatty acid oxidation enzymes allowing cells to switch
to alternative oxidative pathways promoting cell survival. (27)

Tumour growth also depends on formation of new blood vessels (angiogenesis) to facilitate delivery of oxygen and nutrients to the tumour. NR4A receptors are downstream targets of VEGF, promoting proliferation of endothelial cells in vitro and in vivo. (25, 28) Transcription of NR4A receptors is increased by VEGF, hypoxia and CREB activation in vascular endothelial cells. (25) In vivo, the transcriptional activity of NR4A1 and NR4A2 is involved in VEGF mediated angiogenesis. (28, 29)

**NR4A Receptors and Chromosomal Translocations**

Chromosomal translocation of NR4A receptors can lead to oncogenic conversion. For example, translocations involving NR4A3 in extra skeletal myxoid chondrosarcoma, most commonly between Ewing’s Sarcoma Region-1(EWS) and NR4A3 t(9; 22) (q22; q12), resulting in a fusion protein EWS-NOR1, which activates NR4A3 target genes, for example PPAR-γ, and leads to oncogenesis(30)

**CLINICAL-TRANSLATIONAL ADVANCES**

**NR4A Receptors: Identified Roles in Human Cancer**

Altered NR4A receptor expression has been identified in many solid tumours, with a plethora of in vitro roles described. (Table 1) Meanwhile, reduced expression of NR4A1 and NR4A3 receptors is associated with haematological malignancies including acute myeloid leukaemia (AML), and chronic myelodysplastic/myeloproliferative disease. (31)

**Novel Drug Targets**

Drug development targeting NR4A receptor signalling is challenging due to the lack of a natural ligand, their bulky ligand binding domains, the diversity of their cell and context specific functions, and redundancy between members of the NR4A family. Despite these challenges,
several strategies have now been developed to enable therapeutic targeting of NR4A receptor signalling. (1) These include targeting their expression, nuclear export and interaction with co-activators/repressors.

The pro-apoptotic non-genomic action of NR4A1 in the cytoplasm has therapeutic potential and has been manipulated in two ways:

Firstly, by drugs inducing nuclear export of NR4A1. Several drugs already in clinical use are now recognised to induce nuclear export of NR4A1, including 5-Fluorouracil and certain NSAIDS. (15) Novel drugs targeting nuclear export of NR4A1 include n-butyleneephthalide (BP) and Cytosporone B. BP and its derivatives (PCH4) have shown therapeutic potential in Glioblastoma Multiforme (GMB) in vitro and in vivo, and in Oral Squamous Cell Carcinoma (OSCC) in vitro. (32) PCH4 induces apoptosis in OSCC and GBM cell lines in a mechanism dependent on PCH4-mediated increased NR4A1 expression and cytoplasmic translocation. (32, 33) Cytosporone B, a compound isolated from fungi, is a ligand for NR4A1 and induces apoptosis by transactivation of NR4A1 target genes, and by inducing expression and mitochondrial localisation of NR4A1 in vivo and in vitro. (34)

Secondly, a novel nanopeptide (nu-BCP9) has been derived from NR4A1, which mimics the action of NR4A1 on mitochondrial Bcl-2 to promote apoptosis. (14) As the functions of the NR4A receptors are further elucidated, further peptide mimetics may be developed to emulate or block specific actions of the NR4A receptors, at both a non-genomic and transcriptional level.

Another class of drugs which can promote cell death via NR4A receptors are the Diindoylmethane derivatives (CDIMS). CDIMS are unusual as they can cause apoptosis via induction or inhibition of NR4A1 transcriptional activity, in addition to NR4A independent induction of apoptosis via ER stress. The derivative DIM-C-pPhOCH3 (1, 1-bis (3'-indoyl)-1-(p-anisyl) methane is an “activator” of NR4A1 mediated transcription of pro-apoptotic genes e.g. cystathionase, p21 and ATF3, leading to apoptosis in pancreatic cancer cell lines in vitro. Intriguingly, DIM-C-pPOH (1, 1-Bis (3’Indoyl)-1-p-hydroxyphenylmethane), an “inhibitor” of NR4A1 nuclear transactivation also causes apoptosis in pancreatic cancer cell lines, but by reducing transcription of NR4A1-dependent anti-apoptotic and proproliferative genes. (1, 17
This modulation of the opposing effects of NR4A1 on cell survival by CDIMS has potential for selective receptor modulation in pancreatic cancer. Interestingly, in bladder cancer a CDIM (DIM-C-pPhCl) can induce apoptosis and inhibit growth via activation of NR4A2. 

**Chemotherapy Resistance**

NR4A receptors contribute to resistance to chemotherapy; understanding the mechanisms of this resistance may enable therapeutic targeting. Induction of NR4A2 by PGE2 in a cAMP/Protein Kinase A dependent manner promotes resistance to 5-Fluorouracil (5-FU) in squamous cell carcinoma. This has potential relevance for colorectal cancer as PGE2 mediated induction of NR4A2 also occurs in colon cancer cell lines, while 5-FU is a commonly used chemotherapeutic agent in colorectal cancer. Similarly, Riggins et al have suggested roles for NR4A receptors in mediating resistance to doxorubicin in breast cancer. The DNA repair effect of NR4A receptors may contribute to resistance to radiotherapy or chemotherapy (e.g. bleomycin). Manipulation of NR4A/DSB binding may be a strategy to increase tumour sensitivity to chemoradiotherapy.

Other nuclear receptors, like RARβ can influence the expression and function of NR4A receptors and are targetable. For example, by combining a histone deacetylase inhibitor with fenretinide (a synthetic retinoid), the expression, interaction and nuclear export of NR4A1 and RARβ is increased, leading to apoptosis in hepatocellular cell lines in vitro which are otherwise relatively insensitive to fenretinide.

**CONCLUSIONS**

In summary, the study of NR4A receptors represents an exciting new chapter in our understanding of the molecular changes that occur during carcinogenesis. NR4A receptors have diverse cellular effects and function as molecular sensors, which, depending on their cellular microenvironment, may promote, or inhibit cell-death. Targeting expression levels, activity and nuclear export of NR4A receptors, in a tissue dependent manner, in order to manipulate their role in cell survival has potential in the development of novel anti-cancer strategies.
REFERENCES


LEGENDS

Figure 1:
The NR4A receptors are involved in a myriad of cellular functions that contribute to cancer, including angiogenesis, apoptosis, metabolism, migration, proliferation and DNA repair. Expression of the NR4A receptors is induced by a variety of stimuli via multiple cell-signalling pathways including Protein Kinase A/CREB, NFκB, PI3K/AKT, JNK and MAP kinase pathways.

Table 1:
Summary of NR4A receptors in tumours showing altered expression of NR4A receptors in tumour tissue (+=increased, -= decreased) and functional effects of NR4A receptors shown in vitro. Drugs which target NR4A receptors are listed.
NR4A receptors in solid tumours in vitro and in vivo

<table>
<thead>
<tr>
<th>Cancer</th>
<th>NR4A Expression in Tumours</th>
<th>Function in vitro</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon(15, 23, 27, 38)</td>
<td>NR4A1+ NR4A2 +</td>
<td>Apoptosis A1+/-, A2- Proliferation A1 +/-, A2+ Metabolism A2+ Angiogenesis A1+</td>
<td>CDIMS, 5-FU, Butyrate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Migration A1+</td>
<td>NSAIDS/COX-2 inhibitors</td>
</tr>
<tr>
<td>Breast(39-42)</td>
<td>NR4A1+</td>
<td>Apoptosis A1+ A3 + Migration A1-</td>
<td>Nu-BCP9, Retinoids, CDIMS</td>
</tr>
<tr>
<td>Melanoma (19, 43, 44)</td>
<td>NR4A3+</td>
<td>Damage repair A1 + Apoptosis A1, A2+, A3 +, Angiogenesis A1+</td>
<td>CDIMS</td>
</tr>
<tr>
<td>Bladder(4, 45)</td>
<td>NR4A1 + NRA2 +</td>
<td>Apoptosis A1+, A2+/-, Migration A2+</td>
<td>n-butyllephathalide (BP)</td>
</tr>
<tr>
<td>Liver(20, 46)</td>
<td>NR4A1+ (HCC), NR4A2-(mets)</td>
<td>Apoptosis A1+ DNA Repair A1</td>
<td>CDIMS</td>
</tr>
<tr>
<td>Thyroid(47)</td>
<td>NR4A1- , NR4A3-</td>
<td>Apoptosis A1+ A3+</td>
<td>Lithium</td>
</tr>
<tr>
<td>Lung(1, 48-50)</td>
<td>NR4A1+ (NSCLT)</td>
<td>Proliferation A1+/- , Apoptosis A1 +/- , Paraneoplastic (SCLT-ACTH)</td>
<td>CDIMS, Retinoids, e.g. CD437</td>
</tr>
<tr>
<td>Glioblastoma Multiforme</td>
<td>NR4A1+</td>
<td>Apoptosis A1+</td>
<td>n-butyllephathalide(BP)/PCH4</td>
</tr>
<tr>
<td>Pancreatic(1)</td>
<td>NR4A1+</td>
<td>Apoptosis A1 +/- Proliferation A1 +/-</td>
<td>CDIMS, Cytosporone B</td>
</tr>
<tr>
<td>Prostate (1)</td>
<td></td>
<td>Apoptosis A1+/ Proliferation A1-</td>
<td>CDIMS</td>
</tr>
<tr>
<td>Gastric (51)</td>
<td>NR4A2-</td>
<td>Apoptosis A1+</td>
<td>Cytosporone B, Chenodeoxycholic acid</td>
</tr>
<tr>
<td>Extraskeletal myxoid</td>
<td>T(9;22)q22; q12 NR4A3/EWS5</td>
<td>Apoptosis A3-, Proliferation A3+</td>
<td>CDIMS, Vitamin K2</td>
</tr>
<tr>
<td>Chondrosarcoma(30)</td>
<td></td>
<td>Proliferation A2+, Apoptosis A1+, A2-, Migration A2+</td>
<td>n-butyllephathalide(BP)/PCH4</td>
</tr>
<tr>
<td>Cervical (44)</td>
<td></td>
<td>Apoptosis A1+</td>
<td></td>
</tr>
<tr>
<td>Ovarian (1, 52)</td>
<td></td>
<td>Apoptosis A1+</td>
<td></td>
</tr>
<tr>
<td>Oral Squamous Cell Carcinoma (53)</td>
<td>NR4A2+</td>
<td>Apoptosis A1+, A2-</td>
<td>S-FU(resistance)</td>
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